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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Atty. Dkt. No.: FBRC:004USC1/HYL

Prior Application Examiner:
Mary Tung

BOX PATENT APPLICATION
Assistant Commissioner for Patents
Washington, D.C. 20231

Classification Designation:

Prior Group Art Unit: 1644

REQUEST FOR FILING CONTINUATION APPLICATION UNDER 37 C.F.R. § 1.53(b)

This is a request for filing a continuation application under Rule 53(b) (37 C.F.R. § 1.53(b)) of co-pending prior application Serial No. 08/776,337 filed April 21, 1997, entitled "POLYEPITOPE VACCINES."

☒ 1. Enclosed is a copy of the prior application Serial No. 08/776,337 as originally filed, including specification, claims, drawings, and declaration. The undersigned hereby verifies that the attached papers are a true copy of the prior application as originally filed and identified above, that no amendments (if any) referred to in the declaration filed to complete the prior application introduced new matter therein, and further that this statement was made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code, and that such

willful false statement may jeopardize the validity of the application or any patent issuing thereon.

(a) ☒ The inventorship is the same as prior Application Serial No. 08/776,337.

(b) ☐ Deletion of inventor(s). Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. § 1.63(d)(2) and 1.33(b).

(c) ☒ Priority of foreign patent application numbers PM7079, filed 7/27/94, and PM 1009, filed February 8, 1995 in Australia is claimed under 35 U.S.C. § 119(a)-(e). The certified copy:

☐ is enclosed.

☐ has been filed in the prior Application Serial No.

☒ has not been filed.

☐ 2. The Assistant Commissioner is requested to grant Applicant a filing date in accordance with Rule 1.53, and supply Applicant with a Notice of Missing Parts in due course, in accordance with the provisions of Rule 1.53(f).

☒ 3. Enclosed is a check in the amount of $786.00 to cover the filing fee as calculated below and the fee for any new claims added in the Preliminary Amendment referred to in Part No. 9 below.

CLAIMS AS FILED IN THE PRIOR APPLICATION
LESS CLAIMS CANCELED BELOW

FOR	NUMBER FILED	NUMBER EXTRA	RATE	FEE
Basic Fee ----------				$690.00
Total Claims	21 - 20 =	1 X	$18.00 =	$18.00
Independent Claims	4 - 3 =	1 X	$78.00 =	$78.00
Multiple Dependent Claim(s) ----------				$-0-.00
		TOTAL FILING FEES:		**$786.00**

☐ 4. Applicant is entitled to Small Entity Status for this application.

☐ (a) A small entity statement is enclosed.

☐ (b) A small entity statement was filed in the prior nonprovisional application and such status is still proper and desired.

☐ (c) Small entity status is no longer claimed.

☒ 5. If the check is missing or insufficient, the Assistant Commissioner is hereby authorized to charge any fees under 37 C.F.R. §§ 1.16 to 1.21 which may be required for any reason relating to this application, or credit any overpayment to Fulbright & Jaworski Account No.: 50-1212/10011879/01973.

☐ 6. Enclosed is a copy of the current Power of Attorney in the prior application.

☒ 7. Address all future communications to:

Steven L. Highlander
FULBRIGHT & JAWORSKI, L.L.P.
600 Congress Avenue, Suite 2400
Austin, Texas 78701
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☒ 8. The prior application is presently assigned to The Council of the Queensland Institute of Medical Research, Commonwealth Scientific and Industrial Research Organisation, The University of Melbourne, The Walter and Eliza Hall Institute of Medical Research of Royal Melbourne Hospital, Biotech Austraslia PTY Limited, and CSL Limited.

☒ 9. Enclosed is a preliminary amendment. Any additional fees incurred by this amendment are included in the check at No. 3 above and said fee has been calculated after calculation of claims and after amendment of claims by the preliminary amendment.

☐ 10. Cancel in this application claims _____ of the prior application before calculating the filing fee. (At least one original independent claim must be retained).

☒ 11. Amend the specification by inserting before the first line the sentence: --This is a continuation of co-pending application Serial No. 08/776,337 filed April 21, 1997, which is a 371 nationalization of PCT/AU95/00461, filed July 27, 1995, which claims priority to Australian Patent Application 1009, filed February 8, 1995 and Australian Patent Application 7079, filed July 27, 1994. --

☐ 12. Enclosed are formal drawings.

☐ 13. An Information Disclosure Statement (IDS) is enclosed.

☐ (a) PTO-1449.

☐ (b) Copies of IDS citations.

☒ 14. Transfer the sequence information, including the computer readable form previously submitted in the parent application, Serial No. 08/776,337 filed April 21, 1997, for

use in this application. **Under 37 C.F.R. § 1.821(e),** Applicant states **that the paper copy of the sequence listing in this application is identical to the computer readable copy in parent application Serial No. 08/776,337 filed April 21, 1997. Under 37 C.F.R. § 1.821(f),** Applicant also states **that the information recorded in computer readable form is identical to the written sequence listing.**

☒ 15. Other: Copy of Petition to Revive of Parent Application 08/776,337 filed concurrently herewith.

☒ 16. Return Receipt Postcard (should be specifically itemized).

Respectfully submitted,

Steven L. Highlander
Reg. No. 37,642
Attorney for Applicant

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600 Congress Avenue, Suite 2400
Austin, Texas 78701
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Date: 5/22/02

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
ANDREAS SUHRBIER
SCOTT ANTHONY THOMSON
RAJIV KHANNA
SCOTT RENTON BURROWS
BARBARA ELIZABETH HOWIESON COUPAR
DENIS JAMES MOSS

Serial No.: CONT OF 08/776,337

Filed: Concurrently Herewith

For: POLYEPITOPE VACCINES

Group Art Unit: 1644

Examiner: Mary Tung

Atty. Dkt. No.: FBRC:004USC1/HYL

EXPRESS MAIL MAILING LABEL

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MAILING DATE: May 22, 2000

I hereby certify that this paper or fee is being deposited with the United States Postal Service "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 C.F.R. 1.10 on the date indicated above and is addressed to: Assistant Commissioner for Patents, Washington D.C. 20231

Signature

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Please amend the above-identified patent application as follows:

In the claims:

Please delete claims 1-30 and add the following claims:

-- 31. A polynucleotide comprising a nucleic acid sequence encoding a plurality of CTL epitopes, wherein at least two of the sequences encoding said CTL epitopes are contiguous or

spaced apart by intervening sequences, wherein said intervening sequences do not (i) comprise an initiation codon or (ii) encode naturally occurring flanking sequences of the epitopes.

32. A polynucleotide comprising a nucleic acid sequence encoding a plurality of CTL epitopes, wherein the sequence encoding said CTL epitopes are contiguous.

33. The polynucleotide of claim 31, wherein said polynucleotide encodes two CTL epitopes.

34. The polynucleotide of claim 31, wherein said polynucleotide encodes three CTL epitopes.

35. The polynucleotide of claim 31, wherein said polynucleotide encodes nine CTL epitopes.

36. The polynucleotide of claim 31, wherein said polynucleotide encodes ten CTL epitopes.

37. The polynucleotide of claim 31, further defined as an expression vector.

38. The polynucleotide of claim 37, wherein said vector is selected from the group consisting of a viral vector and a virus-like particle (VLP).

39. The polynucleotide of claim 38, wherein said viral vector is a vaccinia vector.

40. The polynucleotide of claim 38, wherein said viral vector is an avipox virus vector.

41. The polynucleotide of claim 38, wherein said vector is a VLP.

42. The polynucleotide of claim 31, wherein at least one of said CTL epitopes is derived from a pathogen.

43. The polynucleotide of claim 31, wherein said polynucleotide comprises a nucleic acid sequence encoding CTL epitopes derived from a plurality of pathogens.

44. The polynucleotide of claim 42, wherein said pathogen is selected from the group consisting of Epstein Barr Virus, Influenza Virus, Cytomegalovirus, Adenovirus and HIV.

45. The polynucleotide of claim 31, wherein at least one of said epitopes is derived from a tumor protein.

46. The polynucleotide of claim 31, further comprising a nucleic acid sequence encoding a T helper cell epitope, a B cell epitope, or a toxin.

47. The polynucleotide of claim 31, further comprising a nucleic acid sequence encoding a T helper cell epitope.

48. The polynucleotide of claim 31, further comprising a nucleic acid sequence encoding a B cell epitope.

49. The polynucleotide of claim 31, further comprising a nucleic acid sequence encoding a toxin.

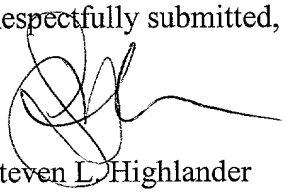
50. A nucleic acid vaccine comprising a polynucleotide comprising a nucleic acid sequence encoding a plurality of CTL epitopes, wherein at least two of the sequences encoding said CTL epitopes are contiguous or spaced apart by intervening sequences, wherein said intervening sequences do not (i) comprise an initiation codon or (ii) encode naturally occurring flanking sequences of the epitopes, and an acceptable carrier.

51. A nucleic acid vaccine comprising a polynucleotide comprising a nucleic acid sequence encoding a plurality of CTL epitopes, wherein the sequences encoding said CTL epitopes are contiguous, and an acceptable carrier. --

A fee as set forth in 37 C.F.R. §§ 1.16-1.21 in the amount of $786.00 is enclosed herewith. If an appropriate check has not been enclosed, or if it is insufficient under 37 C.F.R §§ 1.16 to 1.21, the Commissioner is hereby authorized to deduct any necessary fees from Fulbright & Jaworski Deposit Account No. 50-1212/10011879/01973.

Should Examiner Tung have any questions regarding this communication, she is invited to contact the undersigned at the telephone number listed below.

Respectfully submitted,



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Date: May 22, 2000

POLYEPITOPE VACCINES

The present invention relates to vaccines containing a plurality of cytotoxic T lymphocyte epitopes and to polynucleotides including sequences encoding a plurality of cytotoxic T lymphocyte epitopes.

CD8 + αβ cytotoxic T lymphocytes (CTL) recognise short peptides (epitopes, usually 8-10 amino acids long) associated with specific alleles of the class I major histocompatability complex[1] (MHC). The peptide epitopes are mainly generated from cytosolic proteins by proteolysis, a process believed to involve the multicatalytic proteosome complex[2-7]. Peptides of appropriate length are transported into the endoplasmic reticulum where specific epitopes associate with MHC. The MHC/epitope complex is then transported to the cell surface for recognition by CTL. The influence of sequences flanking CTL epitopes on the proteolytic processing of these epitopes remains controversial[8-12]. However, by constructing a recombinant vaccinia coding for an artificial polypeptide protein containing nine CD8 CTL epitopes in sequence, the present inventors have determined that the natural flanking sequences of CTL epitopes are not required for class I processing, that is each epitope within the polyepitope protein was always efficiently processed and presented to appropriate CTL clones by autologous polyepitope vaccinia infected target cells.

Accordingly, in a first aspect, the present invention consists in a recombinant polyepitope cytotoxic T lymphocyte vaccine, the vaccine comprising at least one recombinant protein including a plurality of cytotoxic T lymphocyte epitopes from one or more pathogens, wherein the at least one recombinant protein is substantially free of sequences naturally found to flank the cytotoxic T lymphocyte epitopes.

Preferably, the at least one recombinant protein does not include any sequences naturally found to flank the cytotoxic T lymphocyte epitopes. However, it should be understood that small lengths (e.g. 1-5 amino acids) of sequences naturally found to flank the cytotoxic T lymphocyte epitopes may be included. The phrase "substantially free of sequences naturally found to flank the cytotoxic T lymphocyte epitopes" is to be taken as including such short lengths of flanking sequences.

In a second aspect, the present invention consists in a polynucleotide, the polynucleotide including at least one sequence encoding a plurality of cytotoxic T lymphocyte epitopes from one or more pathogens, and wherein the at least one sequence is substantially free of sequences encoding peptide sequences naturally found to flank the cytotoxic T lymphocyte epitopes.

Again, it is to be understood that "substantially free of sequences encoding peptide sequences naturally found to flank the cytotoxic T lymphocyte epitopes" includes the possibility of including short peptide (e.g 1-5 amino acids) sequences naturally found to flank the cytotoxic T lymphocyte epitopes.

In a third aspect, the present invention consists in a nucleic acid vaccine, the vaccine comprising the polynucleotide of the second aspect of the present invention and an acceptable carrier.

In a fourth aspect, the present invention consists in a vaccine formulation, the vaccine comprising the recombinant protein of the first aspect of the present invention and an acceptable carrier and/or adjuvant.

In a preferred embodiment of the present invention the at least one recombinant protein includes, and the at least one sequence encodes, at least three cytotoxic T lymphocyte epitopes.

In a further preferred embodiment, the epitopes are from multiple pathogens.

It is also envisaged that the vaccines according to the invention may include immunomodulatory compounds (such as cytokines), other proteins/compounds (such as melittin or regulatory proteins) and/or adjuvants. The vaccines may also include helper epitopes/CD4 epitopes and proteins, B-cell epitopes or proteins containing such epitopes, for example tetanus toxoid. Another example of a vaccine according to the invention comprises a recombinant vaccine construct wherein the polytope including the CTL epitopes is linked to an extracellular glycoprotein or glycoproteins containing B-cell and/or CD4 epitopes.

The vaccines according to the invention may be delivered by various vectors, for example vaccinia vectors, avipox virus vectors, bacterial vectors, virus-like particles (VLP's) and rhabdovirus vectors or by nucleic acid vaccination technology. As polytope proteins are likely to be sensitive to proteolysis during manufacture and/or serum following injection, we envisage that such vaccines may best be delivered using nucleic acid vaccination technologies[12], vector systems or adjuvant systems which protect the polytope protein from proteolysis. Additional information regarding vectors may be found in Chatfield *et al*, Vaccine 7, 495-498, 1989; Taylor *et al*, Vaccine 13, 539-549, 1995; Hodgson "Bacterial Vaccine Vectors" in Vaccines in Agriculture.

A polytope vaccine according to the invention may also include a large number of epitopes (e.g. up to 10 or more) from one pathogen so that the HLA diversity of the target population is covered. For instance a vaccine containing epitopes restricted by HLA A1, A2, A3, A11 and A24 would cover about 90% of the Caucasian population.

A polytope vaccine according to the invention may also be constructed such that the multiple epitopes are restricted by a single HLA allele.

In a preferred embodiment of the fourth aspect of the present invention the vaccine formulation includes ISCOMs. Information regarding ISCOMs can be found in Australian patent No 558258, EP 019942, US4578269 and US4744983, the disclosures of which are incorporated herein by reference.

In order that the nature of the present invention may be more clearly understood preferred forms thereof will now be described with reference to the following examples and accompanying Figures in which:-

Figure 1. Construction of a recombinant vaccinia that expresses a synthetic DNA insert coding for the polytope protein, which contains nine CTL epitopes in sequence. Boxed sequences representing linear B cell epitopes.

Figure 2. CTL recognition of each epitope expressed in the recombinant polytope vaccinia construct.

Figure 3. Polytope vaccinia can recall epitope specific responses. Bulk effectors from donors (A) CM - A24, A11, B7, B44; (B) YW - A2, B8, B38 and (C) NB - A2, A24, B7, B35 were generated by infecting peripheral blood mononuclear cells (PBMC) with the polytope vaccinia at a MOI of 0.01 for 2 hours followed by 2 washes. After 10 days culture in 10% FCS/1640 RPMI the bulk effectors were used against autologous phytohaemagglutinin T cell blasts target cells (E:T 20:1) sensitised with the indicated peptide (10μM) in a standard 5 hour chromium release assay[14]. Bulk effectors generated by the addition of irradiated autologous A type LCLs[14] (LCL to PBMC ratio 1:50) gave similar results to that shown above.

Figure 4 shows the construction of a polytope DNA insert including ten murine CTL epitopes.

Figure 5 shows the sequence of the polytope DNA insert of Figure 1.

Figure 6 provides results of CTL assays conducted on splenocytes harvested from mice vaccinated with recombinant vaccinia including the DNA insert of Figure 3.

Figure 7 shows comparison of spleen MCMV titres (± standard error) 5 weeks after polytope vaccinia vaccination and 4 days after MCMV challenge. P values - unpaired student t-test

Figure 8 DNA vaccination with different plasmids in Balb/c mice.

Figure 9. Murine Polytope vaccinia immunised (IP) mice from these strains (Balb/c, CBA, C56BL/6) had the spleens removed and splenocytes restimulated with peptide (eg for A and A'). effectors were generated by stimulation with influenza NP peptide "TYQRTRALV"). The effectors were then used on peptide coated targets (A-J), virus infected targets (A'-J') and tumour targets (I'). Virus infected targets were either infected (A', F'), with allantoic fluid as negative control or murine polytope vaccinia (Vacc Mu PT) (B'-D', F'-J'), with human polytope vaccinia (Vacc Hu PT) as the negative control.

Example 1

Nine, class I restricted, CTL epitopes from several Epstein-Barr virus nuclear antigens (EBNA) have previously been defined using CTL clones[10, 18-20]. The clones were isolated from a panel of normal healthy Epstein-Barr virus (EBV) seropositive donors and were restricted by different HLA alleles (Table 1). A recombinant polyepitope vaccinia (polytope vaccinia), which coded for a single artificial protein containing all nine of these CTL epitopes, was constructed (see Fig. 1). The DNA sequence coding for this protein was made using splicing by overlap extension (SOEing) and the polymerase chain reaction (PCR) to join six overlapping oligonucleotides. The insert was cloned into pBluescript II, checked by sequencing and transferred into pBCBO7[15] behind a vaccinia promoter to generate

pSTPT1. This plasmid was then used to generate the polytope vaccinia virus by marker-rescue recombination[16]. The artificial polytope protein expressed by this vaccinia therefore containing no sequences naturally found to flank the CTL epitopes in their proteins of origin (Fig. 1).

CTL CLONES	COGNATE EPITOPES	SOURCE	HLA RESTRICTION	REFS
LC13	FLRGRAYGL	EBNA3	B8	13
LC15	QAKWRLQTL	EBNA3	B8	14
CS31	EENLLDFVRF	EBNA6	B44	15
YW22	SVRDRLARL	EBNA3	A0203	14
CM4	KEHVIQNAF	EBNA6	B44	13
NB26	YPLHEQHGM	EBNA3	B3501	14
LX1*	HLAAQGMAY	EBNA3	?	14
JSA2	DTPLIPLTIF	EBNA3	B51#/A2	13
CM9	IVTDFSVIK	EBNA4	A11	16

Table 1: Description of the CTL clones, their cognate epitopes, the proteins of origin (source) and their HLA restriction. The first two letters of the clones refer to the donors. *Not tested (see Fig. 2). #Recent evidence suggests this epitope may be restricted by HLA-B51. All the epitopes have been minimalised except EENLLDFVRF and DTPLIPLTIF.

A DNA sequence coding for the polytope amino acid sequence was designed with codons most frequently used in mammals and incorporated a Kozac sequence[13] and a BamHI site upstream of the start codon. Six 70mer oligonucleotides overlapping by 20 base pairs were spliced together using Splicing by Overlap Extension (SOEing)[14] in 20µl reactions containing standard PCR buffer, 2mM $MgCl_2$, 0.2mM dNTPs, 1.5U of Taq polymerase (hot start at 94°C) using the following thermal program: \94°C for 10 seconds, 45°C for 20 seconds and 72°C for 20 seconds (40 cycles). Half of each gel purified dimer sample was combined in a second PCR reaction (12 cycles) with the addition of 0.5µl of α^{32}p dCTP. The reaction was run on a 6% acrylamide gel and a slice corresponding to the position of the hexamer product was isolated. Two 20mer

oligonucleotides were used to PCR amplify the hexamer using an annealing temperature of 56°C and 25 cycles. The gel purified fragment was cloned into the EcoRV site of pBluescript II KS-, was checked by sequencing and cloned behind the vaccinia P7.5 early/late promoter using the BamHI/SalI sites in the vaccinia plasmid vector pBCBO7[15] to generate pSTPT1. Construction of a TK- recombinant virus was carried out using marker rescue combination between pSTPT1 and VV-WR-L929 as described previously[16]. Plaque purified virus was tested for TK phenotype and for appropriate genome arrangement by Southern blotting of viral DNA[16].

To establish whether each epitope could be efficiently processed from the polytope protein the polytope vaccinia was used to infect a panel of target cells, which expressed the HLA alleles restricting each epitope. Autologous CTL clones specific for each epitope were then used as effector cells in standard chromium release assays. In all cases tested the CTL clones recognised and killed the HLA matched target cell infected with the polytope vaccinia and the appropriate (see Table 1) EBNA vaccinia (positive controls), but not the TK-vaccinia (negative controls) (Fig. 2).

Figure 2 shows CTL recognition of each epitope expressed in the polytope vaccinia construct. Effector CTL are listed in Table 1 (E:T ratio 5:1). Target cells (see below) were infected with recombinant vaccinia expressing either (i) the EPV nuclear antigen (EBNA) recognised by the CTL clone (see Table 1) (positive control), (ii) TK- (negative control), or (iii) the polytope construct (i.e., Polytope vaccinia). Vaccinia infection of the target cells was at a multiplicity of infection of 5:1 followed by 14-16 hour incubation at 37°C prior to use in the standard, 5 hour, ^{51}Cr-release assay[29]. Clone LX1 was no longer available at the time of assay. Target cells; there are two types of EBV, A and B-

type, whose EBNA protein sequences differ significantly. CTL clones LC13, LC15, CM4, NB26, JSA2 and CM9 recognise cells transformed with A-type EBV but not B-type EBV, and CTL clones CS31 and YW22 recognise cells transformed with A-type EBV and EBV[10, 18-20]. The target cells used for the A-type specific CTL were therefore autologous lymphoblastoid cell lines transformed with B-type virus (B-type LCLs). The target cell for CS31 and YW22 were EBV negative B cell blasts, established using anti-CD40 antibody and rIL-4[21].

An additional series of experiments used the polytope vaccinia to stimulate *in vitro* a secondary CTL response from peripheral blood mononuclear cells (PBMC) obtained from healthy EBV seropositive donors. The resulting bulk CTL cultures were then used as effectors against peptide epitope sensitised autologous PHA blasts in a standard chromium release assay. The polytope vaccinia was capable of recalling CTL responses which were specific for epitopes restricted by the HLA alleles expressed by each donor (Fig. 3).

Figure 3 shows that polytope vaccinia can recall epitope specific responses. Bulk effectors from donors (A) CM - A24, A11, B7, B44; (B) YW - A2, B8, B38 and (C) NB - A2, A24, B7, B35 were generated by infecting peripheral blood mononuclear cells (PBMC) with the polytope vaccinia at a MOI of 0.01 for 2 hours followed by 2 washes. After 10 days culture in 10% FCS/1640 RPMI the bulk effectors were used against autologous phytohaemagglutinin T cell blasts target cells (E:T 20:1) sensitised with the indicated peptide (10μM) in a standard 5 hour chromium release assay[19]. Bulk effectors generated by the addition of irradiated autologous A type LCLs[19] (LCL to PBMC ratio 1:50) gave similar results to that shown above.

Two linear B cell epitopes (STNS and NNLVSGPEH) recognised by monoclonal antibodies (8G10/48[22] and

8E7/55[23] respectively) were incorporated at each end of the polytope construct (Fig. 1) to follow the expression of the polytope protein. Western blotting and indirect immunofluorescence antibody staining of polytope vaccinia infected lymphoblastoid cell lines (LCLs) and the processing defective T2 cell line[6,7] using these antibodies failed to detect polytope protein products (data not shown). Recombinant proteins expressed by vaccinia using the same P7.5 promoter are usually readily detected[24] implying that the polytope protein was rapidly degraded in the cytoplasm of mammalian cells. This degradation was not dependent on LMP2 and 7 since the T2 cell line does not express these proteosome associated endopeptidases[6,7]. This phenomenon is consistent with other studies expressing truncated proteins or peptides in mammalian cells[25] and is likely to reflect the inability of such proteins to fold into any secondary or tertiary structures.

A glutathione S-transferase fusion expression vector containing the human polytope was constructed. The DNA fragment coding for the human polytope was excised from pBSpolytope using BamHI/HincII and cloned into the BamHI/AmaI restriction sites in pGex-3x (GST Gene Fusion System Pharmacia) to make pFuspoly. This plasmid was used to express the polytope fusion in bacteria using the standard induction protocols. An aliquot of the bacteria was lysed in loading buffer and run on a 20% SDS PAGE gel with size markers. This gel indicated that the expected protein of approximately 38kD (the human polytope plus the GST domain (26kD)) was being expressed in bacteria containing the plasmid. Western blotting with the two monoclonal antibodies 8G10/48 and 8E7/55 demonstrated that the fusion detected contained the human polytope which has the two linear B cell epitopes (STNS and NNLVSGPEH respectively) incorporated at each end of the polytope

construct. This protein may be incorporated into liposomes or ISCOMs.

Attempts to purify the fusion protein using the GST purification employing glutathione agarose beads failed due to the lack of fusion protein in the bacterial extract supernatant. All the fusion protein precipitated with the cell debris. The protocol was not at fault since GST expressed by itself in a different bacterial culture was in the bacterial extract supernatant and could be purified easily. These data suggest the fusion protein is rapidly degraded in the bacteria unless sequestered into bacterial inclusion bodies from which purification using the GST system is difficult.

Example 2

MATERIALS AND METHODS

Construction of a recombinant vaccinia expressing the murine polytope protein. Ten class I murine CTL epitopes from various diseases were selected so that there were two epitopes for each of H-2Db, H-2Kb, H-2Kd, H-2Kk and H-2Ld which are represented in three strains of mice (see Table 2). These amino acid sequences were arranged such that each of the first 5 epitopes was restricted by a different HLA allele followed by the second group 6-10. The two groups of epitopes were converted to a DNA sequence using the universal codon usage data. These two DNA sequences were separated by an SpeI and flanked by a Xbal restriction site at the 5' end and a AvrII site at the 3' end. Also incorporated at the 5' end is a BamHI restriction site, a Kozac sequence[13] and a methionine start codon. While at the 3' end there is a B cell epitope from *Plasmodium falciparum*, a stop codon and a SalI restriction site see Figures 4 and 5. Five 75mer oligonucleotides and a 76mer oligonucleotide overlapping

by 20 base pairs, representing this 341 base pair sequence, were spliced together using Splicing by Overlap Extension (SOEing)[14] and the polymerase chain reaction (PCR). Primer dimers were made of primers 1 and 2, 3 and 4, 5 and 6 (0.4µg of each) in 40µl reactions containing standard 1x *Pfu* PCR buffer, 0.2 mM dNTPs and 1U of Cloned *Pfu* DNA polymerase (hot start at 94ºC) using a Perkin Elmer 9600 PCR machine programmed with the following thermal program; 94ºC for 10 seconds, 42ºC for 20 seconds and 72ºC for 20 seconds for 5 cycles. At the end of 5 cycles the PCR programme was paused at 72ºC and 20µl aliquots of reactions 2 and 3 were mixed (reaction 1 was left in the PCR machine) and subjected to a further 5 cycles. At cycle 10 the program was paused again and 20µl of reaction 1 added to the combined reactions 2 and 3 and a further 5 cycles completed. The combined 40µl sample was then gel purified on a 4% Nusieve agarose gel (FMC) and a gel slice corresponding to the correct sized fragment removed and spun through Whatmann 3MM paper. Two 20mer oligonucleotides were used to PCR amplify the full length product using the standard reaction mix as above and an annealing temperature of 50ºC and 25 cycles. The full length PCR fragment was gel purified in a 4% Nusieve agarose gel, cloned into the EcoRV site of pBluescript IIKS$^-$ to make pBSMP and checked for mutations by sequencing. The DNA insert of a plasmid containing the correct sequence was excised using BamHI/SalI restriction enzymes and cloned, using the same enzymes, behind the vaccinia P7.5 early/late promoter in the plasmid shuttle vector pBCB07[15] to generate pSTMOUSEPOLY. Construction of a TK- recombinant virus was carried out using marker rescue recombination between pSTMOUSEPOLY and VV-WR-L929 using protocols described previously[16]. Plaque purified virus was tested for TK phenotype and for appropriate genome arrangement by Southern blotting of viral DNA[17].

Vaccination of mice with recombinant murine polytope vaccinia. The recombinant vaccinia was used to vaccinate 3 mice in each of the 3 strains of mice Balb/cv, C57BL/6 and CBA. The vaccinations were I.V. 50µl containing 5×10^7 pfu of vaccinia and the mice were left to recover for three weeks. The TK- vaccinia was used as a negative control for each strain of mouse in this experiment.

Cytotoxic T cell assays. Splenocytes were harvested from the vaccinated mice 3 weeks post vaccination and restimulated with the appropriate peptides (1µg/ml) in vitro[16]. No peptide were used for restimulations as negative controls. After 7 days of culture the restimulated bulk effectors were harvested and used in a 5 hour, ^{51}Cr-release assays. The targets used in these assays were ConA blasts generated from each of the strains coated with one of the peptides presented by that strain. Three effector to target ratios were used 50:1, 10:1 and 2:1 the results are shown in Figure 6.

RESULTS

Construction of murine recombinant polytope vaccinia, The list of epitopes included in the murine polytope are listed in Table 2.

Table 2 CTL epitopes of the murine CTL polytope

SOURCE	SEQUENCE	RESTRICTION	MOUSE STRAIN
Influenza nuclear protein (366-374)	ASNENMDAM	H-2D^b	C57BL/6
Adenovirus 5 E1A (234-243)	SGPSNTPPEI	H-2D^b	C57BL/6
Ovalbumin (257-264)	SIINFEEKL	H-2K^b	C57BL/6
Sendai virus nuclear protein (324-332)	FAPGNYPAL	H-2K^b	C57BL/6
Influenza nuclear protein (147-155)	TYQRTRALV	H-2K^d	Balb/c
P. Berghei Circumsporozoite protein (249-257)	SYIPSAEKI	H-SK^d	Balb/c
Influenza nuclear protein (50-58)	SDYEGRLI	H-2K^k	CBA
Influenza NS1 (152-160)	EEGAIVGEI	H-SK^k	CBA
Murine Cytomegalovirus pp89 (168-176)	YPHFMPTNL	H-2L^d	Balb/c
Lymphocytic choriomeningitis virus nuclear protein (118-126)	RPQASGVYM	H-SL^d	Balb/c

The construction of the polytope DNA insert is summarised in Fig. 4. The polytope sequence is shown in Fig 5.

CTL assays.

Each epitope in the polytope induced a primary CTL response in mice with the appropriately MHC allele. No competition between two epitopes restricted by the same allele was observed. (the high flu NP response in CBA mice given TK- controls is likely to be due to a naturally acquired influenza).

Polytope constructs containing multiple CTL epitopes from various pathogens restricted by various MHC alleles

are clearly capable of generating primary CTL responses to each epitope within the polytope vaccine. This has clear application in all vaccines where CTL responses are required for protection. For instance, multiple HIV CTL epitopes might be combined in a therapeutic vaccine to foreshadow epitopes expressed by escape mutants and thereby prevent disease progression.

Murine polyepitope mice have SIINFEKL specific CTL which can kill the ovalbumin transfected cell line EG7 in vitro and in vivo.

SIINFEKL specific CTL which kill the EG7 tumor cells demonstrated in vitro

Spleen cells from murine vaccinia immunised mice were collected 4 weeks post vaccination and restimulated in vitro with 10ug/ml SIINFEKL for 7 days. Effectors could not lyse the untransfected parent line EL4 but could lyse the EG7 tumour cells and EL4 cells sensitised with SIINFEKL..

Protection against EG7 tumour in vivo afforded by murine polytope

Mice (C57B6) were given either human polytope vaccinia (Thomsom et al., 1995) or murine polytope vaccinia (10^7 pfu/mouse/ip) and 4 weeks later received 10^7 EL4 or EG7 tumour cells (Moore et al., 1988. Cell 54,777) subcutaneously (10 or 11 mice per group).

The number of mice with visable tumours (all were >1cm diameter) at day 9 is given.

Human Polytope Vaccinia		Murine Polytope Vaccinia	
EG7	EL4	EG7	EL4
10/10*	10/10	0/11	10/10

*(Two mice had tumours <1cm in diameter)

Protection against MCMV

BALB/c mice were challenged with MCMV (K181 strain, 8 x 10^3 PFU, 100µl intraperitoneally) 5 weeks after polytope vaccinia vaccination. Four days after challenge the viral titres per gram of spleen were determined the results are shown in Fig.7 (method of Scalzo *et al*)[17].

Evaluation of polytope vaccines delivered in a DNA plasmid.

The polytope protein described above contained a linear antibody epitope recognised by a monoclonal antibody. As described above the polytope protein could not, however, be detected in cells infected with the polytope vaccinia indicating that it is very unstable; a likely consequence of having no folding structure. It was thus considered that delivery of a polytope vaccine may be best achieved using nucleic acid vaccination technology or with an adjuvant system that protects from proteolysis (eg liposomes or ISCOMs).

The CMV promotor cassette from pCIS2.CXXNH (Eaton *et al* (1986) *Biochemistry* 25(26) p8343) was cloned into the EcoRI site of pUC8 in the same orientation as the LacZ gene to make the plasmid pDNAVacc (used as a control plasmid in the DNA vaccination experiments). This plasmid then had the murine polytope (from pBSMP) inserted into the XhoI site in the multiple cloning site to form pSTMPDV. The plasmid pRSVGM/CMVMP has fragments sourced from a number of different plasmids. The RSV promotor was excised from pRSVHygro (Long *et al* (1991) *Hum. Immunol.* 31, 229-235), the murine GM-CSF gene from pMPZen(GM-CSF) (Johnson *et al* (1989) *EMBO* 8, 441-448) and the CMV promotor cassette from pCS (Kienzie *et al* (1992) *Arch. Virol.* **124** p123-132). Into the CMV cassette was the murine polytope cloned into the SmaI site of the multiple

cloning site. Both genes , murine GM-CSF and the murine polytope, use the bi-directional polyA from SV40.

Nine 6 week old female Balb/c mice were injected I.M. with 50µg of either pDNAVacc (plasmid control), pSTMPDV (murine polytope only) or pRSVGM/CMVMP (murine GM-CSF and murine polytope) in 50µl of PBS (see next figure). They were given boosters with another 50µg of the same plasmids at 3 weeks. At 8 weeks from the vaccination these mice were killed and their spleens removed. Splenocytes were isolated and cultured with peptide as previously described for vaccinia vaccinated animals. These bulk effectors were then used in standard ^{51}Cr release assays against P815 cells coated with peptide corresponding to the epitopes in the murine polytope that are presented by Balb/c mice. The assay was done for 6 hours at E:T ratios of 2:1, 10:1 and 50:1.

The results of these experiments are shown in Fig 8.

SPECIFIC CTL ACTIVITY AGAINST PEPTIDE COATED AND VIRUS INFECTED TARGETS INDUCED BY THE MURINE POLYTOPE VACCINIA

Method

1. **Vaccination and Effector Cell Preparation.** Mice (3 per group) were vaccinated intraperitoneally (IP) with 5 x 10^7 PFU vaccinia. Mice were boosted via the same route and with the same amount of vaccinia week 3. The spleens were removed 6 weeks after the initial vaccination and the splenocytes were isolated after erythrocyte lysis with ACK Buffer (0.15M NH_4Cl, 1mM $KHCO_3$, 0.1mM Na_2EDTA)(Current Protocols in Immunology, Ed JE Coligan, AM Kruisbeek, DH Margulies, EM Shevach, W Strober, 1994 John Wiley and Sons Inc. USA.). 5 x 10^6 splenocytes per well were peptide restimulated (1µg/ml) in bulk T cell media (RPMI/10% Fetal Calf Serum (FCS), 2mM Glutamine, $5x10^{-5}M$ 2-Mercaptoethanol) for seven days prior to cytotoxic T

lymphocyte (CTL) assay on ^{51}chromium (^{51}Cr) labelled target cells[17]. The peptides used for restimulation are given above A to J. The effectors were used against either peptide coated targets A-J, viral infected targets (A'-J') or transfected antigen expressing targets (I').

2. **Preparation of Target Cells.** Cell lines used as targets in these assays were P815 for Balb/c (H-2^d), EL-4 and EG7 for C57BL/6 (H-2^b), L929 for CBA (H-2^k) L929, or con A blasts prepared from the Balb/c, C57BL/6 or CBA mice, respectively.[1] To express the required epitope for CTL killing, target cells were either pre-incubated with (i) peptide (A-J), (ii) vaccinia (B'-D', F'-J'). or (iii) Influenza (A', E'), or maintained as the (iv) Ovalbumin-expressing plasmid transfectant of El-4 (EG7) in the case of the SIINFEKL epitope system (I').

(i) Peptide coated targets (A-J): Target cells were centrifuged at 1000rpm/5 min. The supernatant was discarded to approximately 200µg/ml and 10-20µl of either RPMI (No peptide) or 200ug/ml stock peptide in RPMI (peptide coated) (final concentration 10µg/ml) was added to the cell pellet. One hundred microlitres of ^{51}Cr was added to cell pellet and the cells were incubated at 37^{o}C for 1 hr. The cells were then washed twice with RPMI/10%FCS through a FCS underlayer and resuspended at 10^5/ml for target cells in the CTL assay.

(ii) Vaccinia (Vacc.) infected targets (B'-D', F'-J'): Vaccinia used for virus infected targets were the Murine Polytope (Vacc Mu PT), with the Human Polytope (Vacc Hu PT) as the negative control. Cell lines infected by vaccinia were P815 (B'-D'), L929 (F') and EL-4 (G'-J'). The target cells were centrifuged at 1000rpm/5 min. The supernatant was discarded to approximately 200ul and the cells (approx. 10^6 cells) infected with vaccinia at a multiplicity of infection (MOI) of 10:1 by adding 20µl

vaccinia (10^9 pfu/ml) followed by incubation for 1 hr at 37°C. Five millilitres of RPMI/10%FCS was then added, cells mixed and incubated overnight at 37°C. These cells were subsequently centrifuged and supernatant discarded into camdyne. One hundred microlitres of ^{51}Cr was added to cell pellet and the cells incubated at 37°C for 1 hr. The cells were then washed twice with RPMI/10%FCS through a FCS underlayer and resuspended at 10^5/ml for target cells in the CTL assay.

(iii) Influenza infected targets (A', E'): The A/PR/8/34 strain of Influenza virus was used for the Balb/c targets (A') and the reassortant A/Taiwan/1/86 (IVR-40) for the CBA targets (E'). Allantoic fluid was used as the negative control. Cell lines infected by influenza were P815 (A') and L929 (E'). Target cells were centrifuged at 1000rpm/5 min. and supernatant was discarded. Five hundred microlitres: 50µl Influenza virus (10^8/ml EID) or Allantoic Fluid, 50µl ^{51}Cr, 400µl RPMI/1%FCS was added to the cell pellet and incubated for 1 hr at 37°C. Ten millilitres of RPMI/10%FCS was added, mixed and incubated a further 2 hr at 37°C. The cells were then washed twice with RPMI/10%FCS through a FCS underlayer and resuspended at 10^5/ml for target cells in CTL assay.

(iv) Ovalbumin expressing targets (I'): EG7 cells are EL-4 cells transfected with an expression plasmid containing chicken ovalbumin cDNA (Moore MW, Carbone FR and Bevan BJ (1988) Introduction of soluble protein into Class 1 pathway of antigen processing and presentation. *Cell* 54: 777-785.). These cells were maintained in RPMI/10% FCS. 20mM Hepes, 2mM Glutamine, 1mM Na Pyruvate, 1001U/ml penicillin and 100µg/ml Streptomycin. The plasmid was selected and maintained in Geneticin (G-418) at 500µg/ml once per month. EL-4 cells with no peptide

(EL4 no pep) were used as the negative control. The cells were centrifuged at 1000rpm/5 min. and supernatant discarded to approximately 200µl. One hundred microlitres of ^{51}Cr was added to cell pellet and the cells incubated at 37°C for 1 hr. The cells were then washed twice with RPMI/10%FCS through a FCS underlayer and resuspended at 10^5/ml for target cells in the CTL assay.

3. **CTL Assay.** The restimulated splenocytes ($5x10^6$/ml) were dispensed (100µl) in triplicate at three Effector: Target ratios (50, 10, $2x10^4$ effector cells: $1x10^4$ target cells for the CTL assay. One hundred microlitres of target cells (10^5/ml) were added to all effectors and control wells (Spontaneous release = 100µl media; Maximal release = 100µl 0.5% SDS/ RPMI/10%FCS). Microtitre plates were centrifuged at 500rpm for 5 min. and incubated at 37°C for 6hr. Plates were recentrifuged at 500rpm/5 min. and 25µl of supernatant was counted for ^{51}Cr release. Percentage Specific Lysis represents averages of triplicate counts: 100 x (Test cpm - Spontaneous cpm)/(Maximal cpm - Spontaneous cpm).

The results are shown in Figure 9.

DNA vaccination experiment

The initial DNA vaccination experiments illustrate that the polytope can be delivered using DNA vaccination. In addition, that vaccination may be improved by the co-delivery of a cytokine gene (GM-CSF), although in this experiment the improvement is not statistically significant.

The current system is clearly sub-optimal. Likely improvements would be the use of potentially better plasmid vectors e.g. the vectors from Vical, San Diego (Sedegah M, R Hedstrom, P Hobart, SL Hoffman, 1994. Protection against malaria by immunisation with plasmid

DNA encoding circumsporozoite protein. PNAS 91, 9866-9870) and the use of better delivery systems (to IM injection) employing a gene gun (Sun WH., Burkholder JK., Sun J., Culp J., Lu XG., Pugh TD., Ershler WB, Yang NS. IN VIVO CYTOKINE GENE TRANSFER BY GENE GUN REDUCES TUMOUR GROWTH IN MICE. Proceedings of the National Academy of Sciences of the United States of America. 92:2889-2893, 1995.). In addition priming against CTL epitopes usually requires CD4 T cell help[17] thus the inclusion helper epitopes or proteins in the construct may improve the level and reliability of CTL priming by the murine DNA vaccine polytope.

Lack of "Original antigenic sin" or the ability of a polytope to raise immune responses to all the epitopes in a polytope when the individual has already got a response to one of the epitopes.

Introduction

Original antigenic sin is a term given to an antibody based phenomena whereby an existing antibody response to an epitope prevents the raising of an immune response to a second epitope when that epitope is present on the same protein as the first epitope (Benjamini E., Andria M.L., Estin C.D., Notron, F.L. & Leung C.Y. (1988) Studies on the clonality of the response to an epitope of a protein antigen. Randomness of activation of epitope - recognizing clones and the development of clonal dominance. *J. Immunol.* 141,55.). The reason for this phenomena is that large population of primed B cells specific for the first epitope bind and mop up all the available antigen before a naive B cell specific for the second antibody has a chance to bind the antigen, process it and present it to T helper cells. A similar situation might occur when an individual is vaccinated with a polytope when he/she already has a response to one of the

epitopes in the polytope. The existing CTL might kill all the polytope expressing cells before any of the other epitopes can be presented to naive T cells.

Method

To test this possibility mice (Balb/c) were infected with 10^4 pfu of Murine cytomegalovirus (MCMV) (K181 strain - Scalzo et al. 1995) and left for 5 weeks at which point strong CTL responses specific for the MCMV epitope, YPHFMPTNL, had developed (Scalzo et al. 1995 - Fig 2A). These mice were then given the murine polytope vaccinia and spleen cells assayed 10 days later for CTL specific for the three other epitopes presented by the polytope in this strain of mouse (RPQASGVYM, Lymphocytic choriomeningitis virus nuclear protein, H-2L^d; TYQRTRALV, influenza nuclear protein, H-2K^d and SYIPSAEKI, P. Berghei circumsporozoite protein, H-2K^d).

Results

Responses to each of the three new epitopes was observed following polytope vaccination, illustrating that the YPHFMPTNL specific CTL did not prevent priming of CTL specific for RPQASGVYM, TYQRTRALV and SYIPSAEKI when all four epitopes are presented together in the polytope. (Control animals receiving the human polytope vaccinia instead of the murine polytope vaccinia, showed only YPHFMPTNL specific CTL).

This series of experiments illustrate that if a polytope was, for instance, designed to cover a variety of different diseases, an individual receiving such polytope vaccine, but who had already been exposed to one of the target diseases would still be immunised against the remaining CTL epitopes in the polytope.

As will be apparent to those skilled in the art the present inventors have shown that the natural flanking sequences of CTL epitopes are not required for class I processing, that is each epitope within the polyepitope protein was always efficiently processed and presented to

appropriate CTL clones by autologous polyepitope vaccinia infected target cells. It will be apparent to those skilled in the art that the polytopes may include sequences not naturally found to flank the epitopes.

As discuussed above the present invention can be used with a range of epitopes. A range of epitopes are now available on an Internet address which is described in Brusic *et al* Nucleic Acids Research, 1994, 22; 3663-5.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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CLAIMS:-

1. A recombinant polyepitope cytotoxic T lymphocyte vaccine, the vaccine comprising at least one recombinant protein including a plurality of cytotoxic T lymphocyte epitopes from one or more pathogens, wherein the at least one recombinant protein is substantially free of sequences naturally found to flank the cytotoxic T lymphocyte epitopes.

2. A recombinant polyepitope cytotoxic T lymphocyte vaccine as claimed in claim 1 in which the at least one recombinant protein does not include sequences naturally found to flank the cytotoxic T lymphocyte epitopes.

3. A recombinant polyepitope cytotoxic T lymphocyte vaccine as claimed in claim 1 or claim 2 in which recombinant protein includes at least three cytotoxic T lymphocyte epitopes.

4. A recombinant polyepitope cytotoxic T lymphocyte vaccine as claimed in any one of claims 1 to 3 in which the cytotoxic T lymphocyte epitopes are from multiple pathogens.

5. A polynucleotide including at least one sequence encoding a plurality of cytotoxic T lymphocyte epitopes from one or more pathogens, wherein the at least one sequence is substantially free of sequences encoding peptide sequences naturally found to flank the cytotoxic T lymphocyte epitopes.

6. A polynucleotide as claimed in claim 5 in which the at least one sequence is free of sequences encoding peptide sequences naturally found to flank the cytotoxic T lymphocyte epitopes.

7. A polynucleotide as claimed in claim 5 or claim 6 in which the at least one sequence encodes at least three cytotoxic T lymphocyte epitopes.

8. A polynucleotide as claimed in any one of claims 5 to 7 in which the at least one sequence encodes cytotoxic T lymphocyte epitopes are from multiple pathogens.

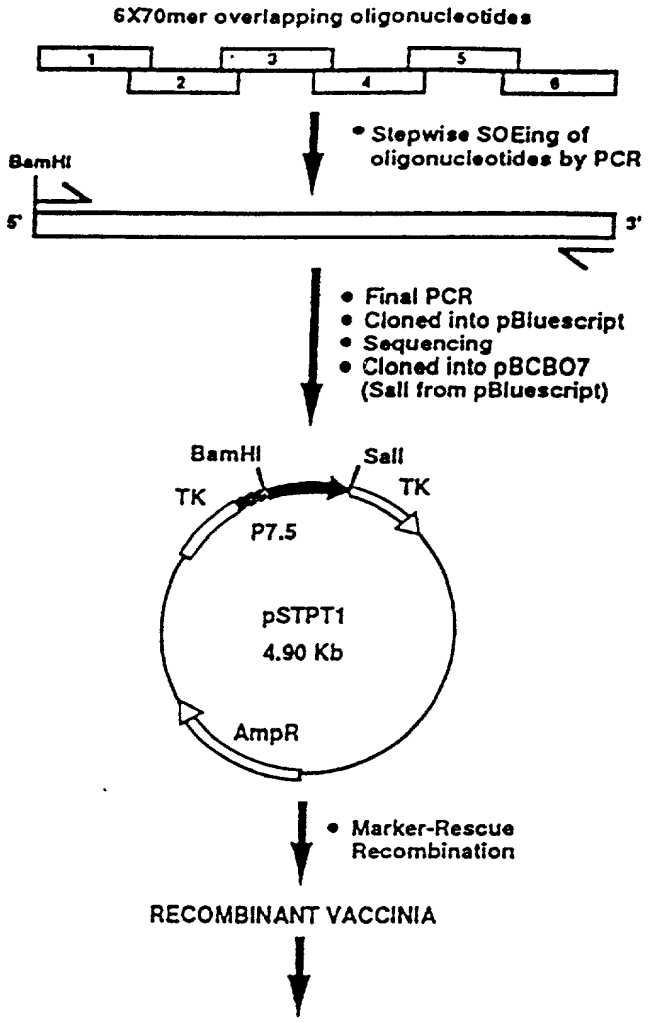
9. A nucleic acid vaccine, the vaccine comprising the polynucleotide as claimed in any one of claims 5 to 8 and an acceptable carrier.

10. A vector including the polynucleotide as claimed in any one of claims 5 to 8.

11. A vector as claimed in claim 10 in which the vector is selected from the group consisting of vaccinia vectors, avipox virus vectors, bacterial vectors, virus-like particles (VLP's) and rhabdovirus vectors.

12. A vaccine formulation, the vaccine formulation comprising the recombinant protein as claimed in any one of claims 1 to 4 and an acceptable carrier and/or adjuvant.

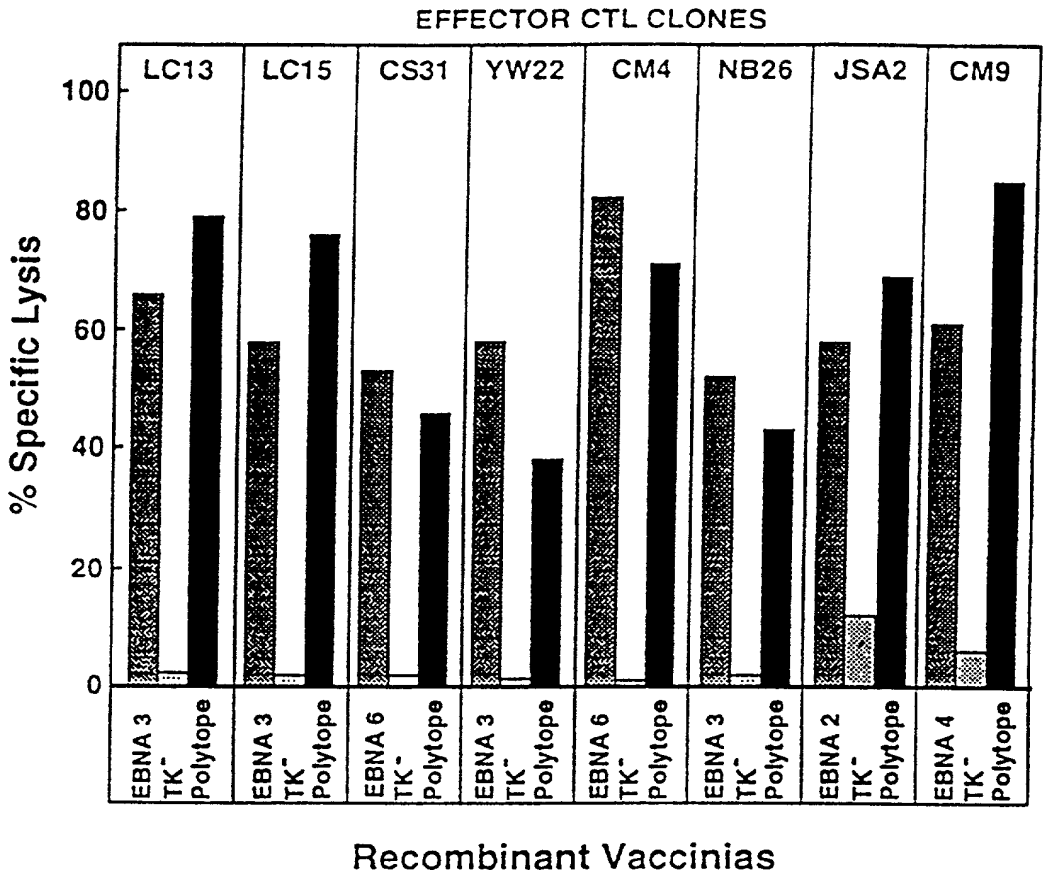
13. A vaccine formulation as claimed in claim 12 in which the formulation includes ISCOMs.



MSTNSFLRGRAYGL QAKWRLQTL-
EENLLDFVRF SVRDRLARLKEHVIQNAF-
YPLHEQHGMHLAAQGMAY DTPLIPLTIF-
IVTDFSVIK NNLVSGPEH

Polytope Protein Sequence

FIGURE 1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LC13
LC15
CS31
YW22
CM4
NB26
JSA2
CM9
% Specific Lysis
100
80
60
40
20
0
EBNA 3 TK⁻ Polytope
EBNA 3 TK⁻ Polytope
EBNA 6 TK⁻ Polytope
EBNA 3 TK⁻ Polytope
EBNA 6 TK⁻ Polytope
EBNA 3 TK⁻ Polytope
EBNA 2 TK⁻ Polytope
EBNA 4 TK⁻ Polytope
Recombinant Vaccinias

FIGURE 2

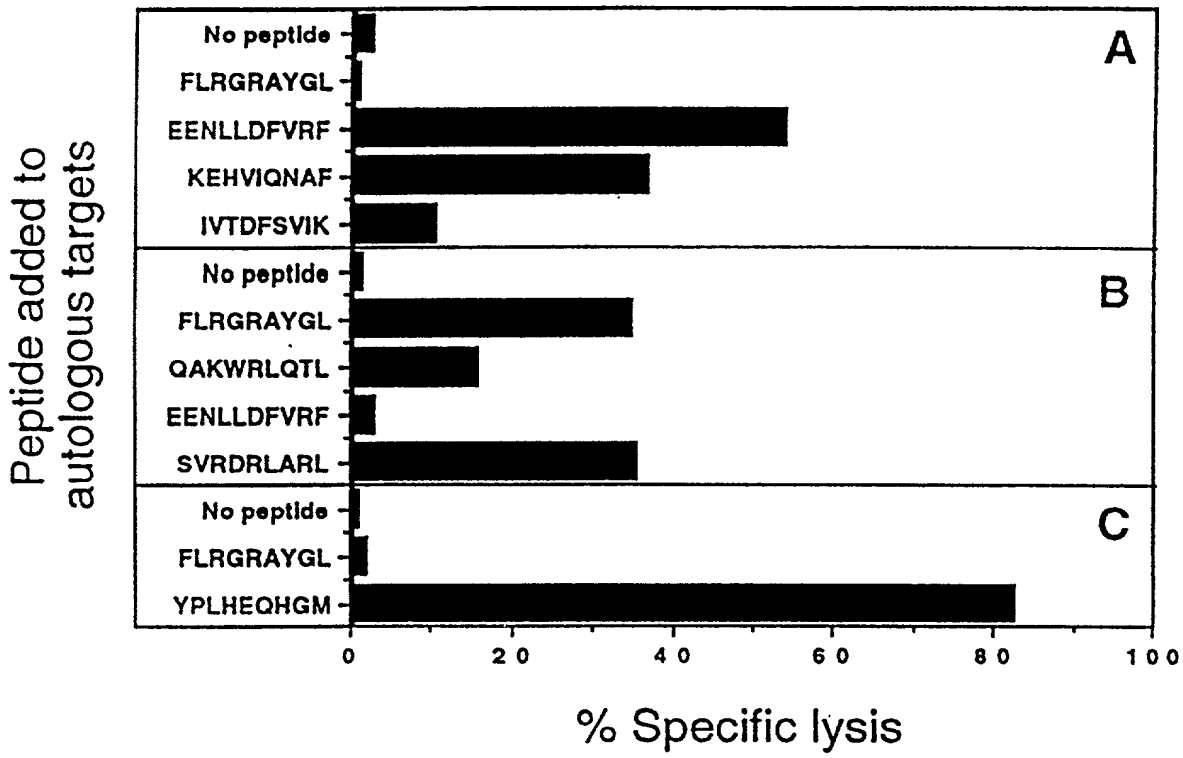

Peptide added to autologous targets
No peptide
FLRGRAYGL
EENLLDFVRF
KEHVIQNAF
IVTDFSVIK
A
No peptide
FLRGRAYGL
QAKWRLQTL
EENLLDFVRF
SVRDRLARL
B
No peptide
FLRGRAYGL
YPLHEQHGM
C
0
20
40
60
80
100
% Specific lysis

FIGURE 3

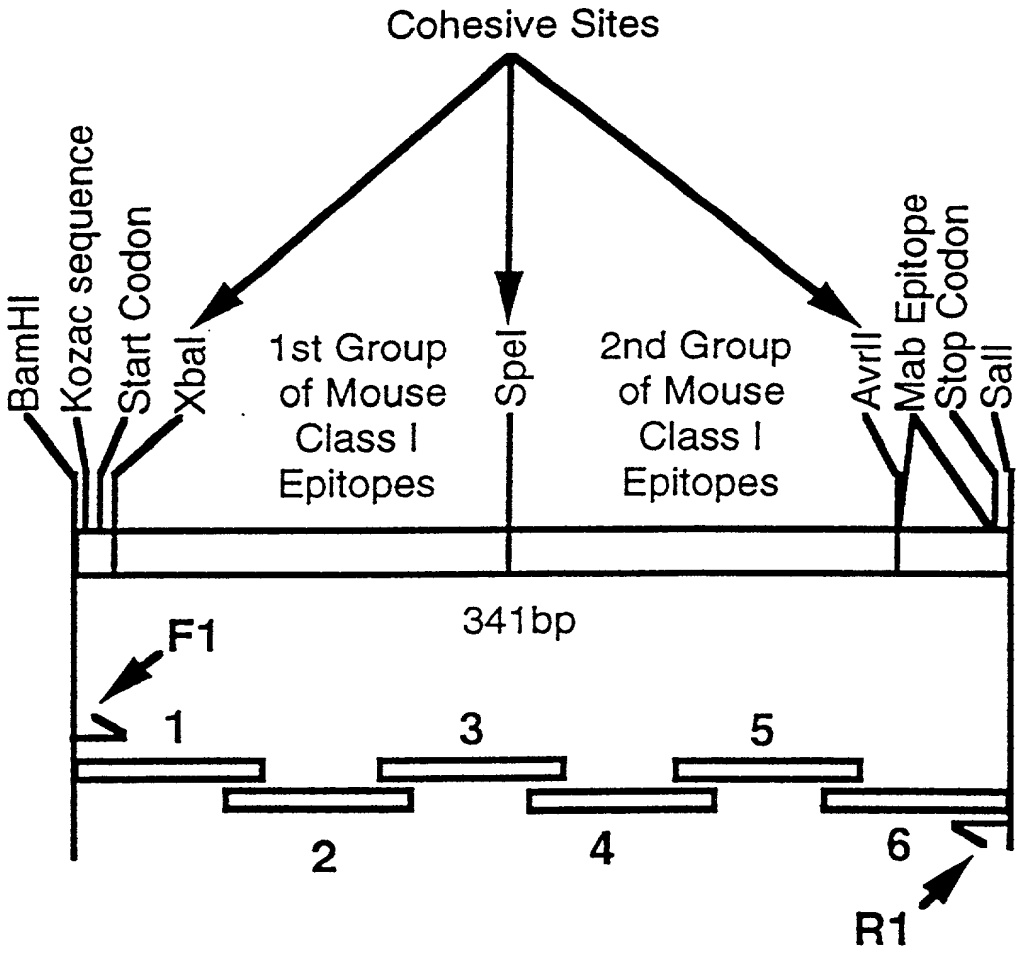

Cohesive Sites
BamHI
Kozac sequence
Start Codon
XbaI
1st Group of Mouse Class I Epitopes
SpeI
2nd Group of Mouse Class I Epitopes
AvrII
Mab Epitope
Stop Codon
SalI
341bp
F1
1
3
5
2
4
6
R1

FIGURE 4

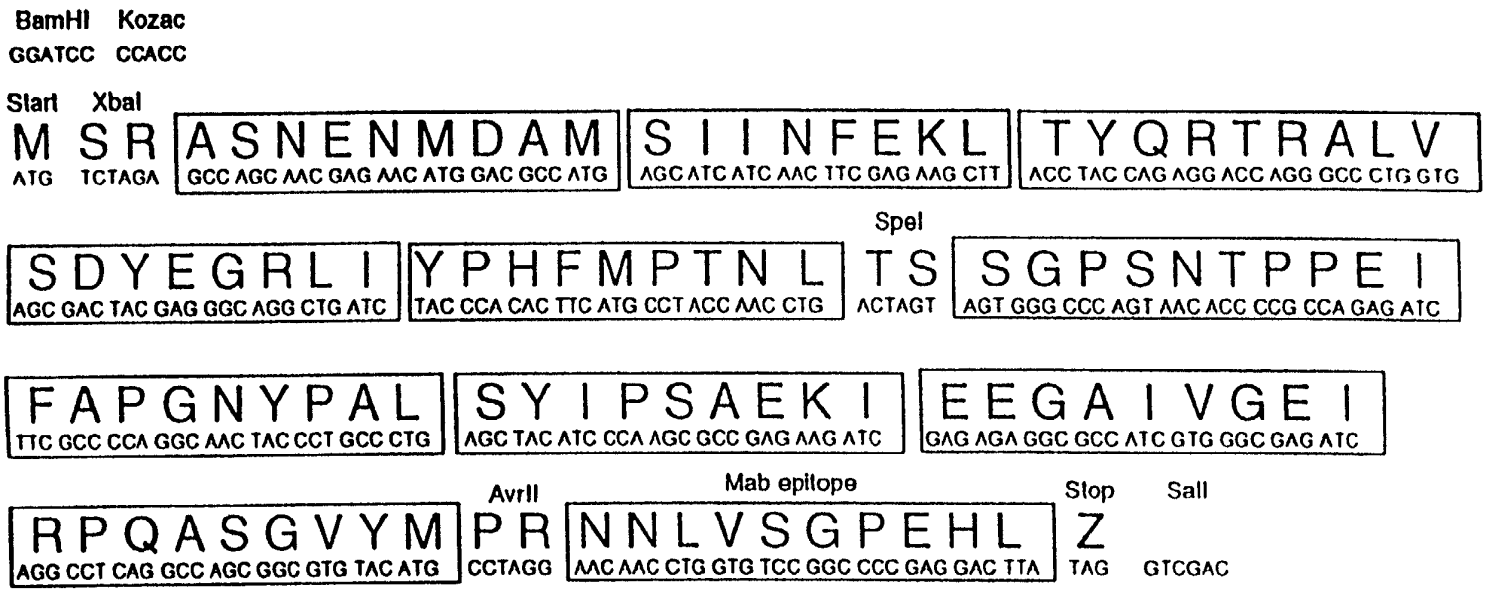

BamHI Kozac
GGATCC CCACC
Start XbaI
M S R A S N E N M D A M S I I N F E K L T Y Q R T R A L V
ATG TCTAGA GCC AGC AAC GAG AAC ATG GAC GCC ATG AGC ATC ATC AAC TTC GAG AAG CTT ACC TAC CAG AGG ACC AGG GCC CTG GTG
SpeI
S D Y E G R L I Y P H F M P T N L T S S G P S N T P P E I
AGC GAC TAC GAG GGC AGG CTG ATC TAC CCA CAC TTC ATG CCT ACC AAC CTG ACTAGT AGT GGG CCC AGT AAC ACC CCG CCA GAG ATC
F A P G N Y P A L S Y I P S A E K I E E G A I V G E I
TTC GCC CCA GGC AAC TAC CCT GCC CTG AGC TAC ATC CCA AGC GCC GAG AAG ATC GAG AGA GGC GCC ATC GTG GGC GAG ATC
AvrII Mab epitope Stop SalI
R P Q A S G V Y M P R N N L V S G P E H L Z
AGG CCT CAG GCC AGC GGC GTG TAC ATG CCTAGG AAC AAC CTG GTG TCC GGC CCC GAG GAC TTA TAG GTCGAC

FIGURE 5

Balb/c

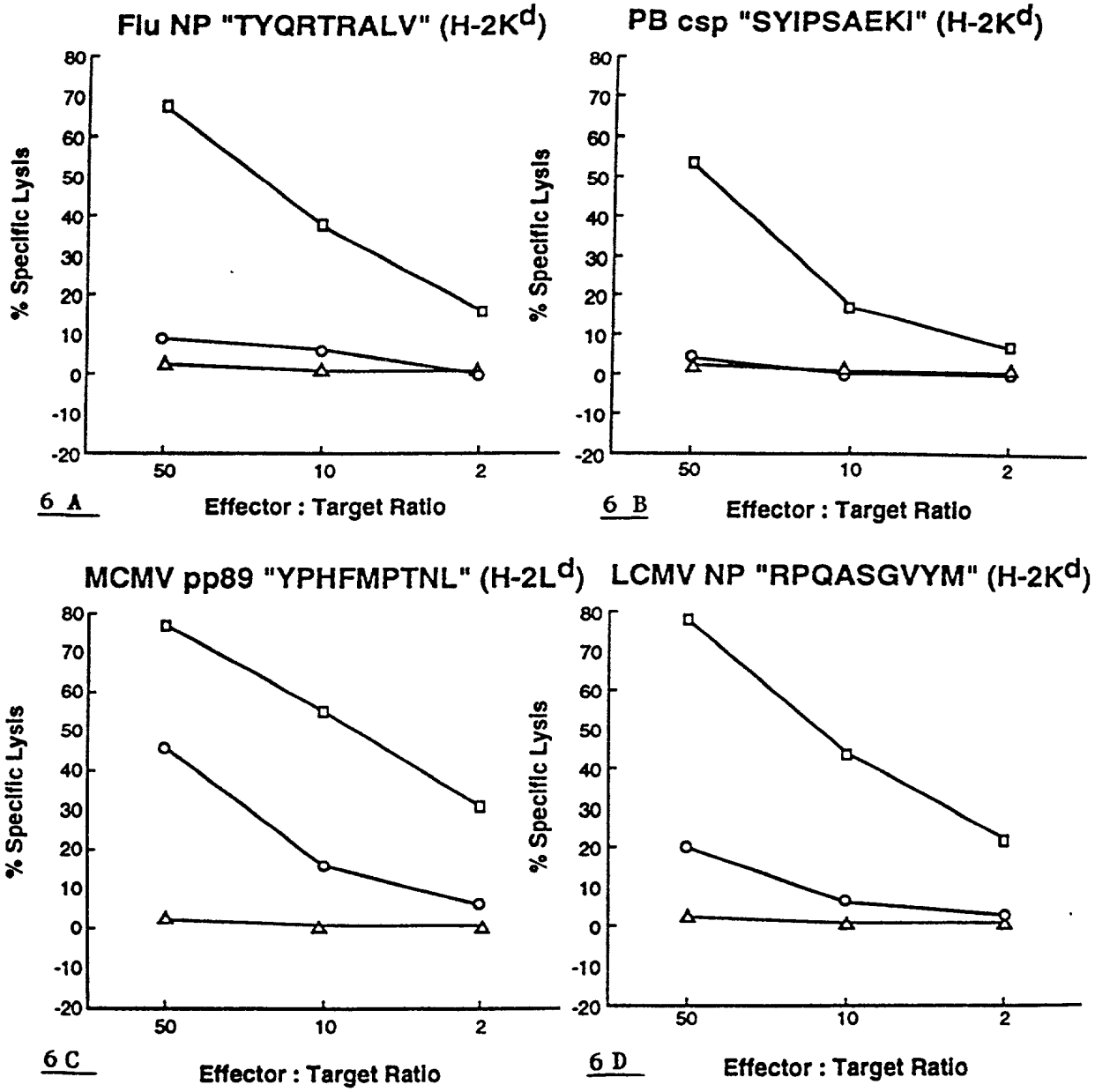
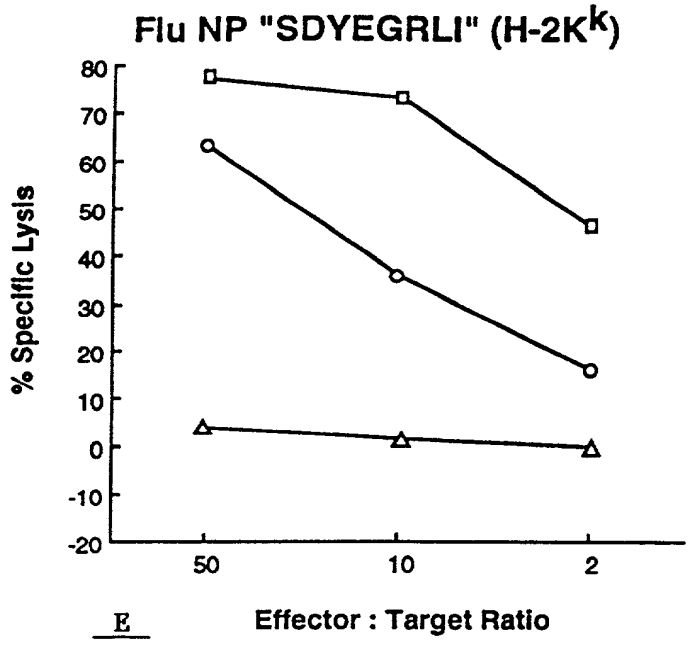
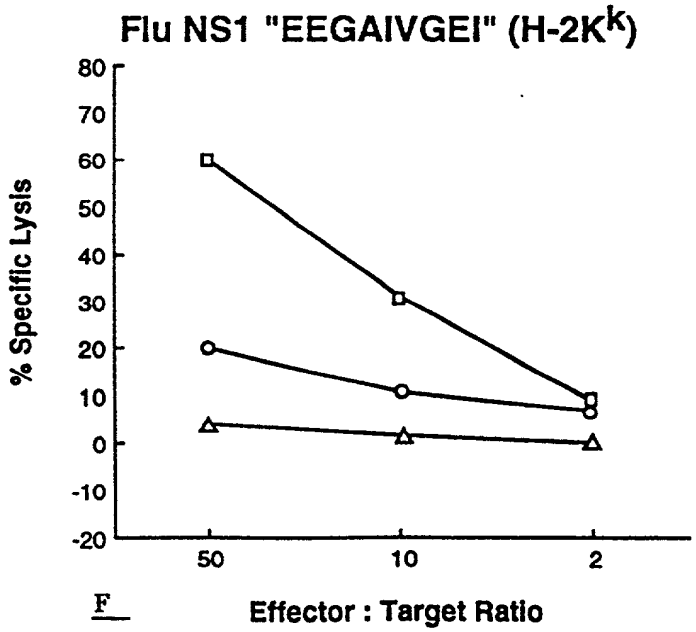


FIGURE 6

CBA



E



F

LEGEND

Bulk Splenocytes from Polytope Vaccinated Mice

No peptide Control

Bulk Splenocytes from TK- Vaccinated Mice

FIGURE 6

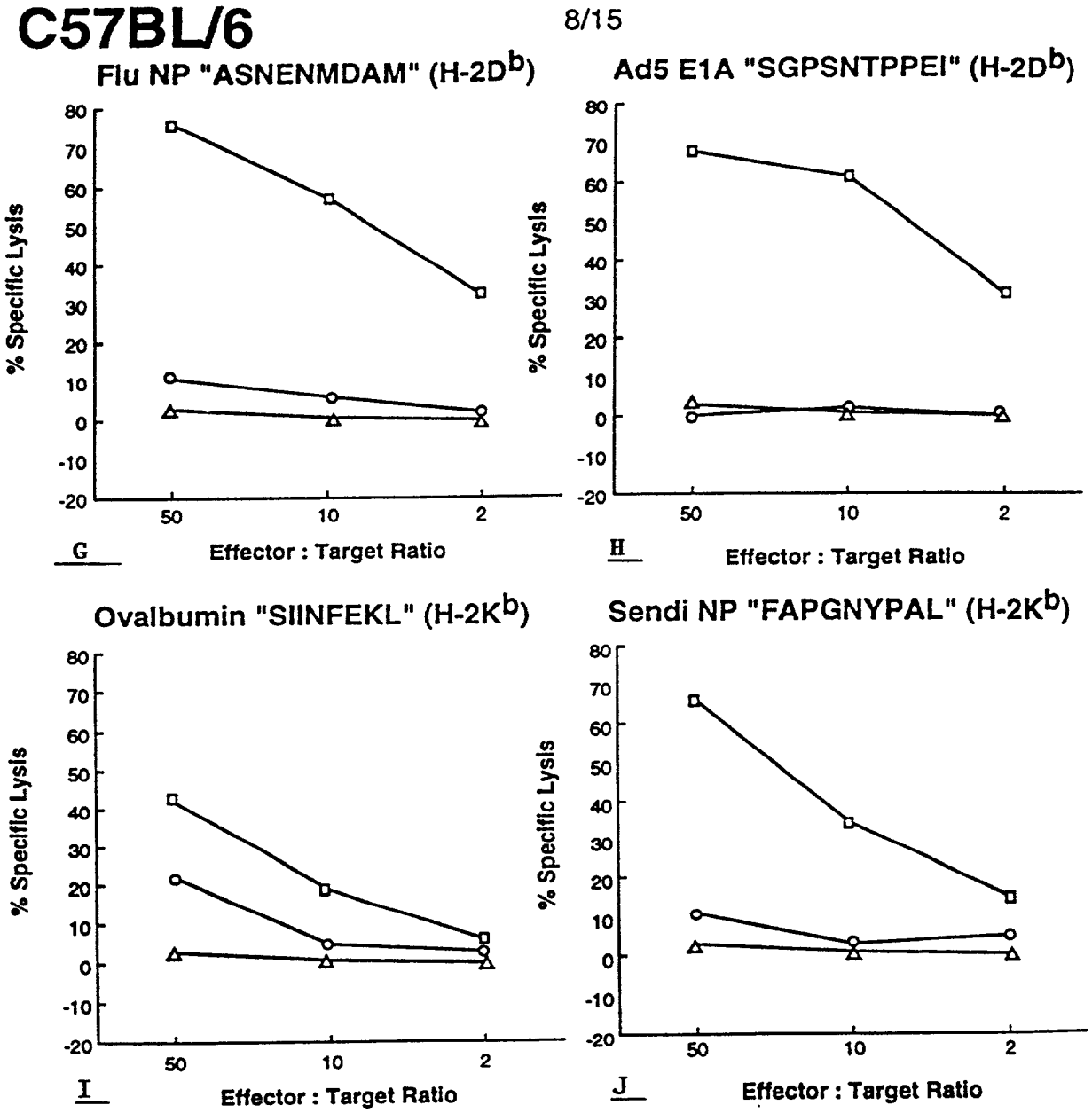

C57BL/6
Flu NP "ASNENMDAM" (H-2D^b)
Ad5 E1A "SGPSNTPPEI" (H-2D^b)
Ovalbumin "SIINFEKL" (H-2K^b)
Sendi NP "FAPGNYPAL" (H-2K^b)
% Specific Lysis
Effector : Target Ratio
50
10
2
G
H
I
J
LEGEND
Bulk Splenocytes from Polytope Vaccinated Mice
No peptide Control
Bulk Splenocytes from TK- Vaccinated Mice

FIGURE 6

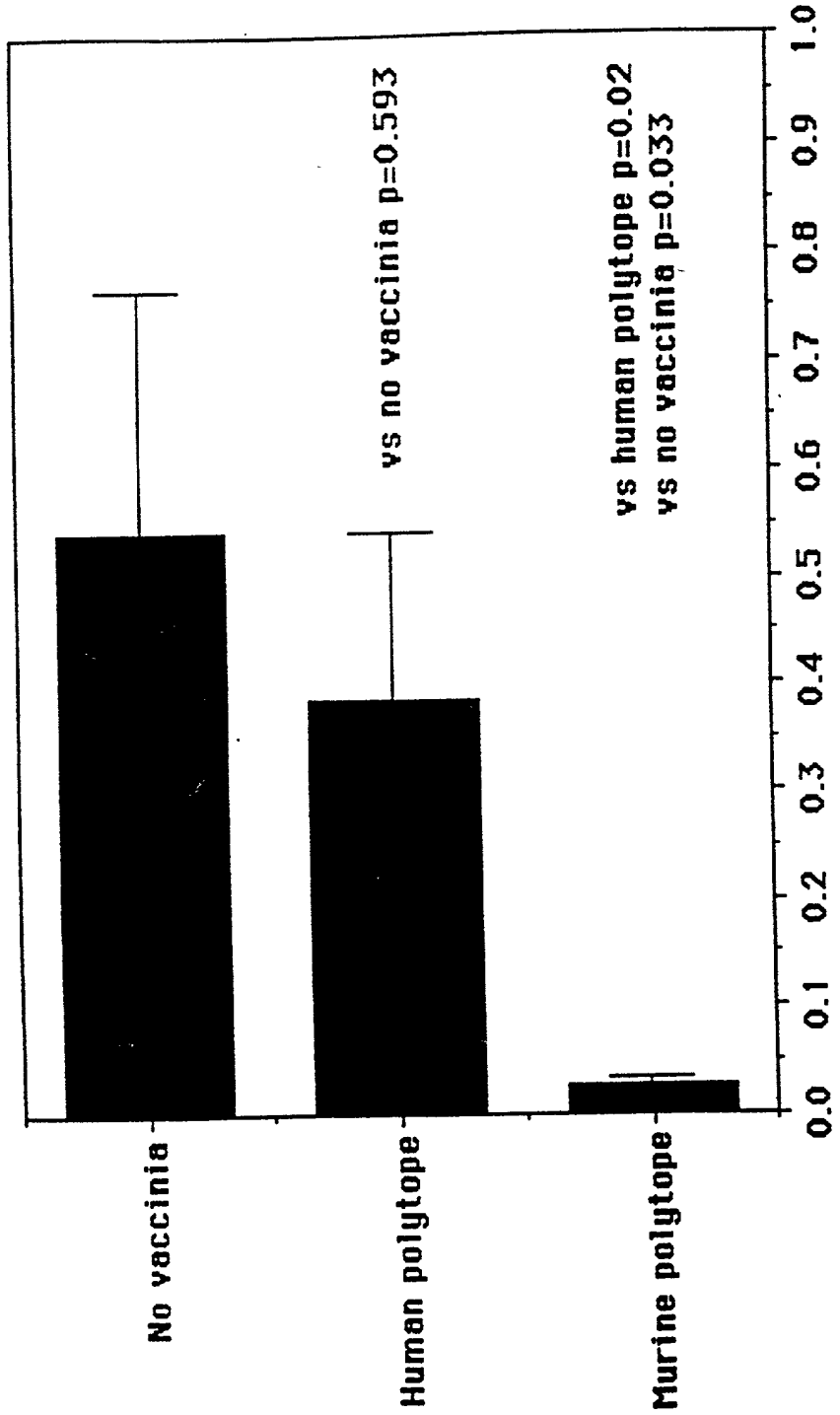

No vaccinia
Human polytope
Murine polytope
vs no vaccinia p=0.593
vs human polytope p=0.02
vs no vaccinia p=0.033
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
10e6 Pfu/g spleen

FIGURE 7

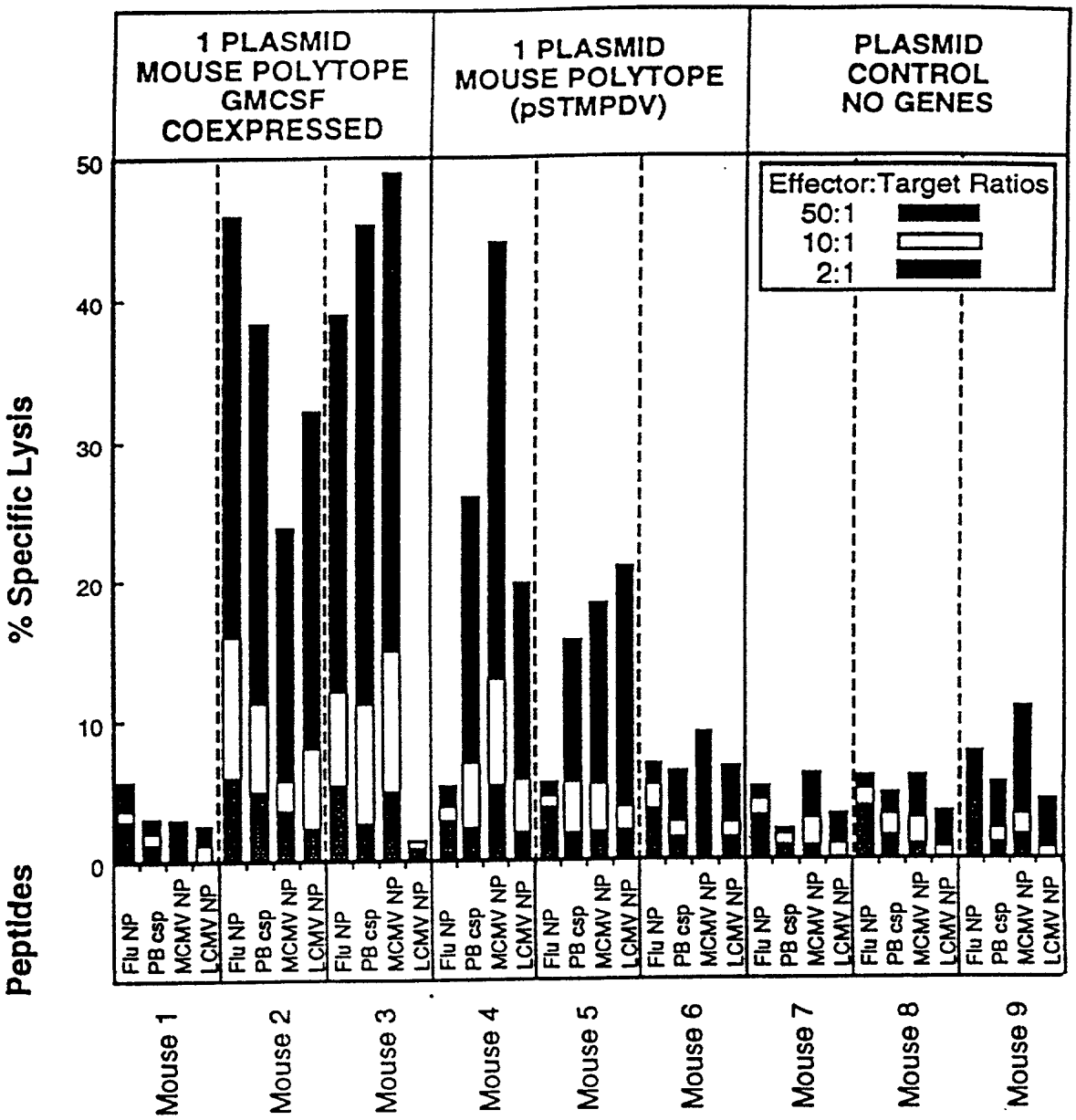

1 PLASMID MOUSE POLYTOPE GMCSF COEXPRESSED
1 PLASMID MOUSE POLYTOPE (pSTMPDV)
PLASMID CONTROL NO GENES
Effector:Target Ratios
50:1
10:1
2:1
% Specific Lysis
0
10
20
30
40
50
Peptides
Flu NP
PB csp
MCMV NP
LCMV NP
Mouse 1
Mouse 2
Mouse 3
Mouse 4
Mouse 5
Mouse 6
Mouse 7
Mouse 8
Mouse 9

FIGURE 8

Balb/c I.P. Vaccinated Mice

PEPTIDE COATED TARGETS VIRUS INFECTED TARGETS

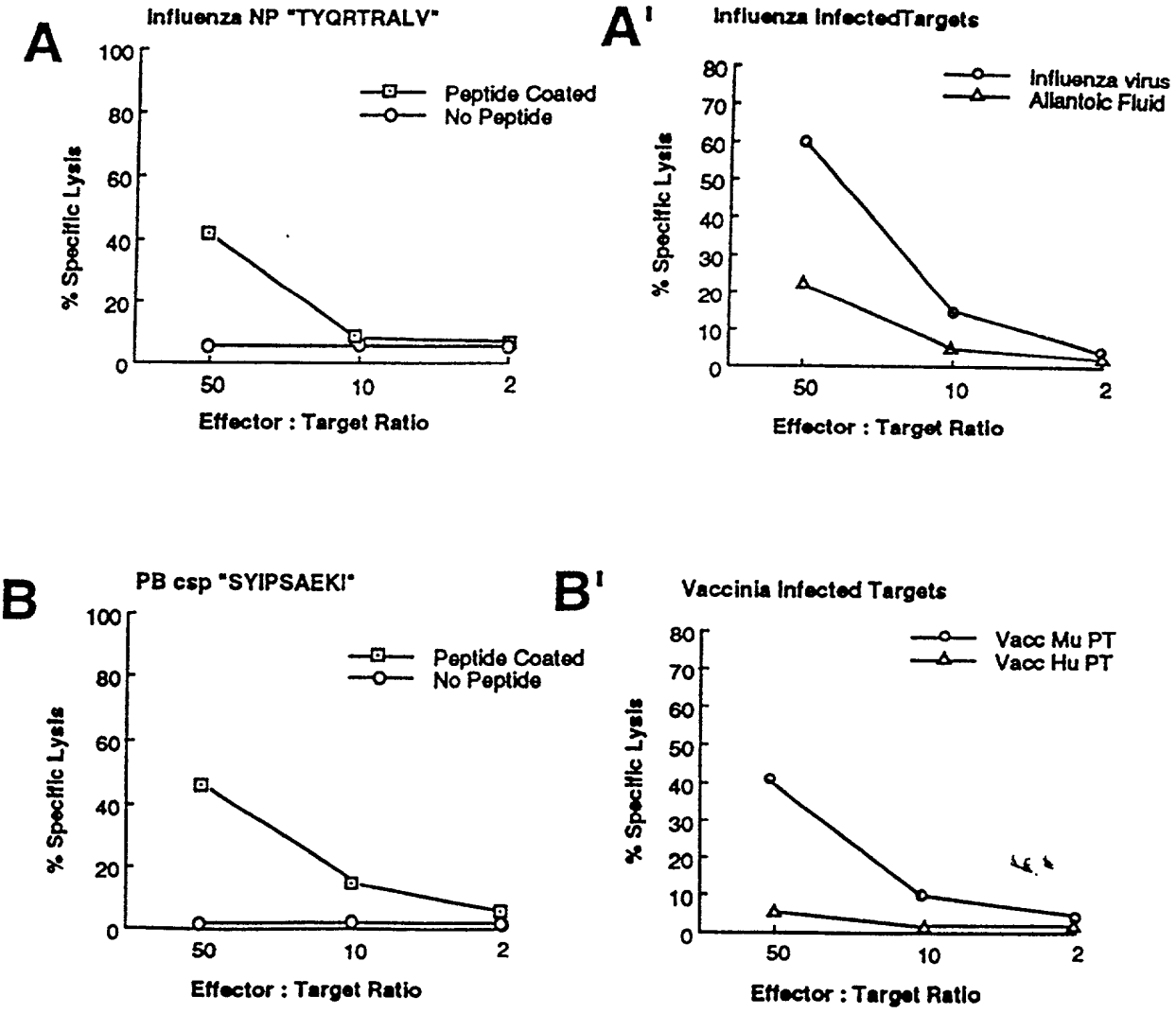


FIGURE 9

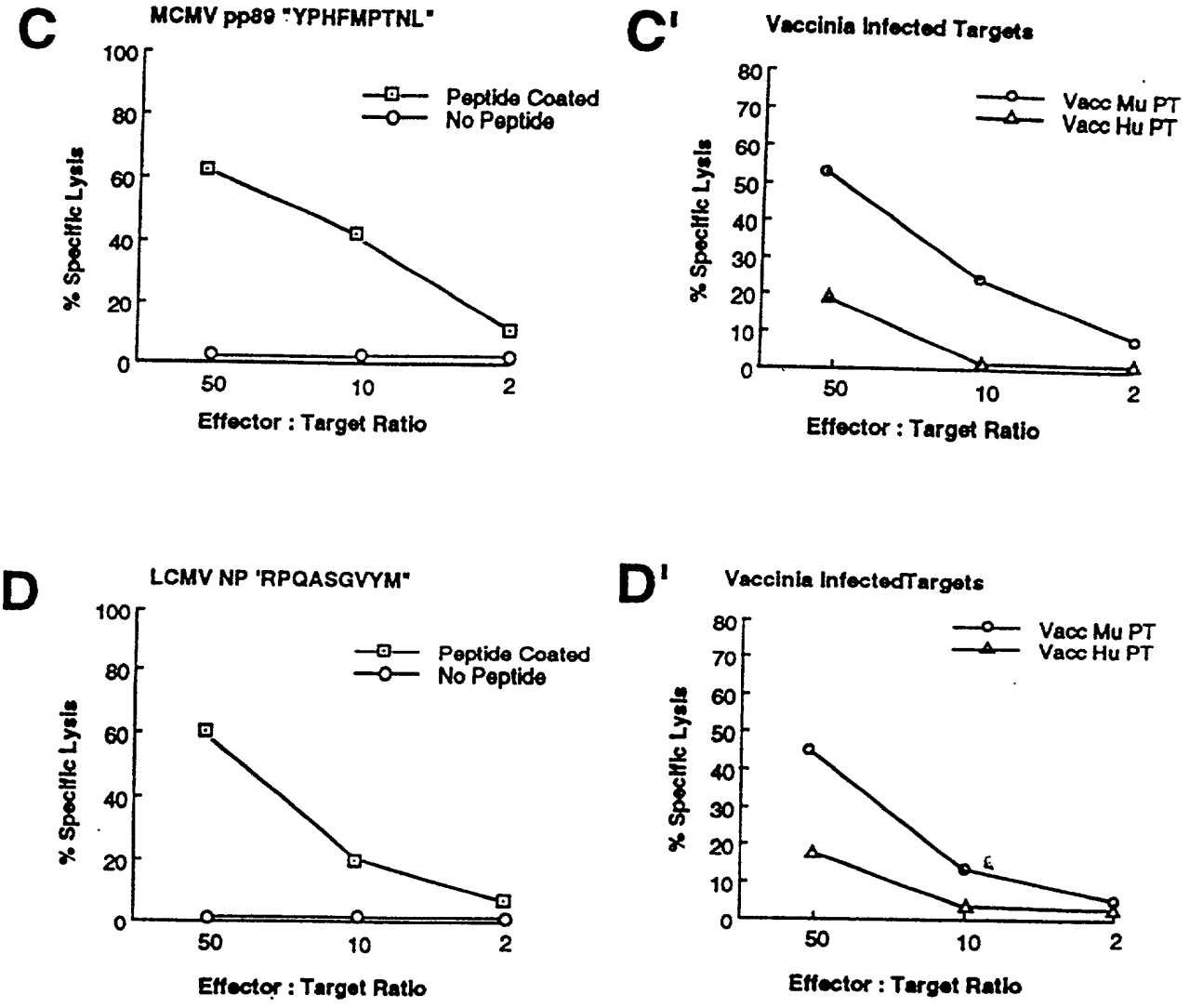

C
MCMV pp89 "YPHFMPTNL"
Peptide Coated
No Peptide
% Specific Lysis
Effector : Target Ratio
C'
Vaccinia Infected Targets
Vacc Mu PT
Vacc Hu PT
% Specific Lysis
Effector : Target Ratio
D
LCMV NP 'RPQASGVYM"
Peptide Coated
No Peptide
% Specific Lysis
Effector : Target Ratio
D'
Vaccinia InfectedTargets
Vacc Mu PT
Vacc Hu PT
% Specific Lysis
Effector : Target Ratio

FIGURE 9

CBA I.P. Vaccinated Mice

PEPTIDE COATED TARGETS VIRUS INFECTED TARGETS

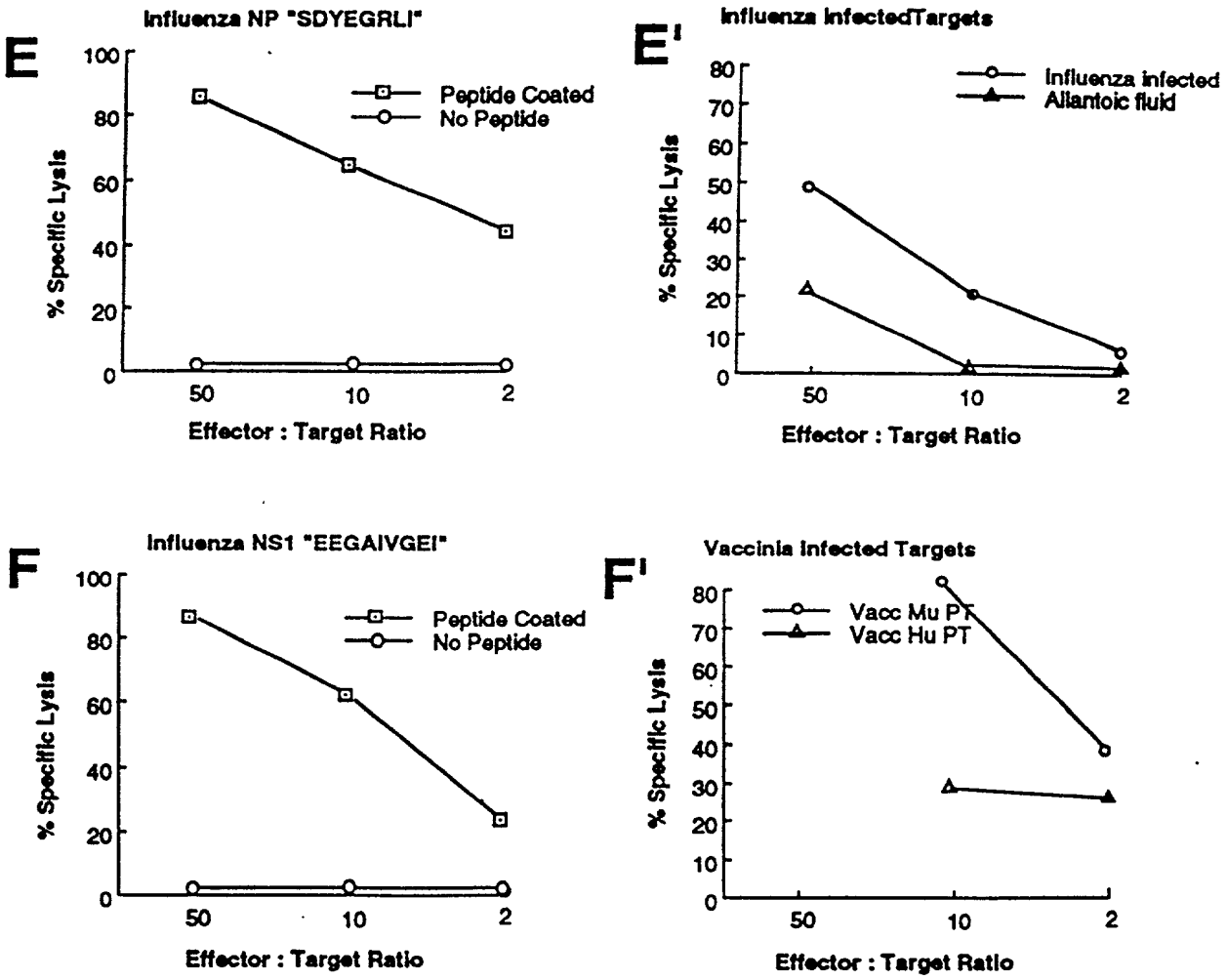


FIGURE 9

C57BL/6 I.P. Vaccinated Mice

PEPTIDE COATED TARGETS

TUMOUR AND VIRUS INFECTED TARGETS

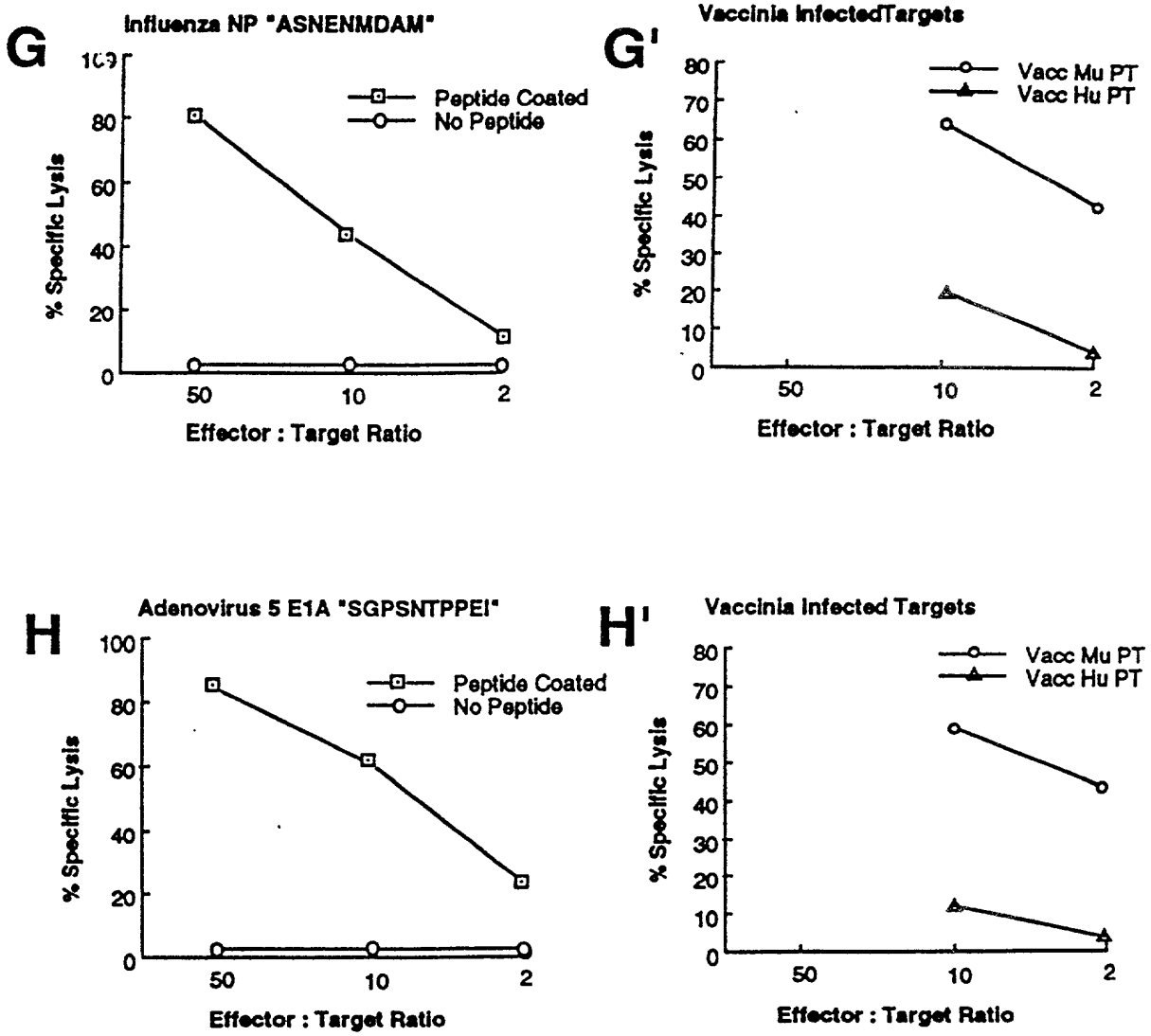


FIGURE 9

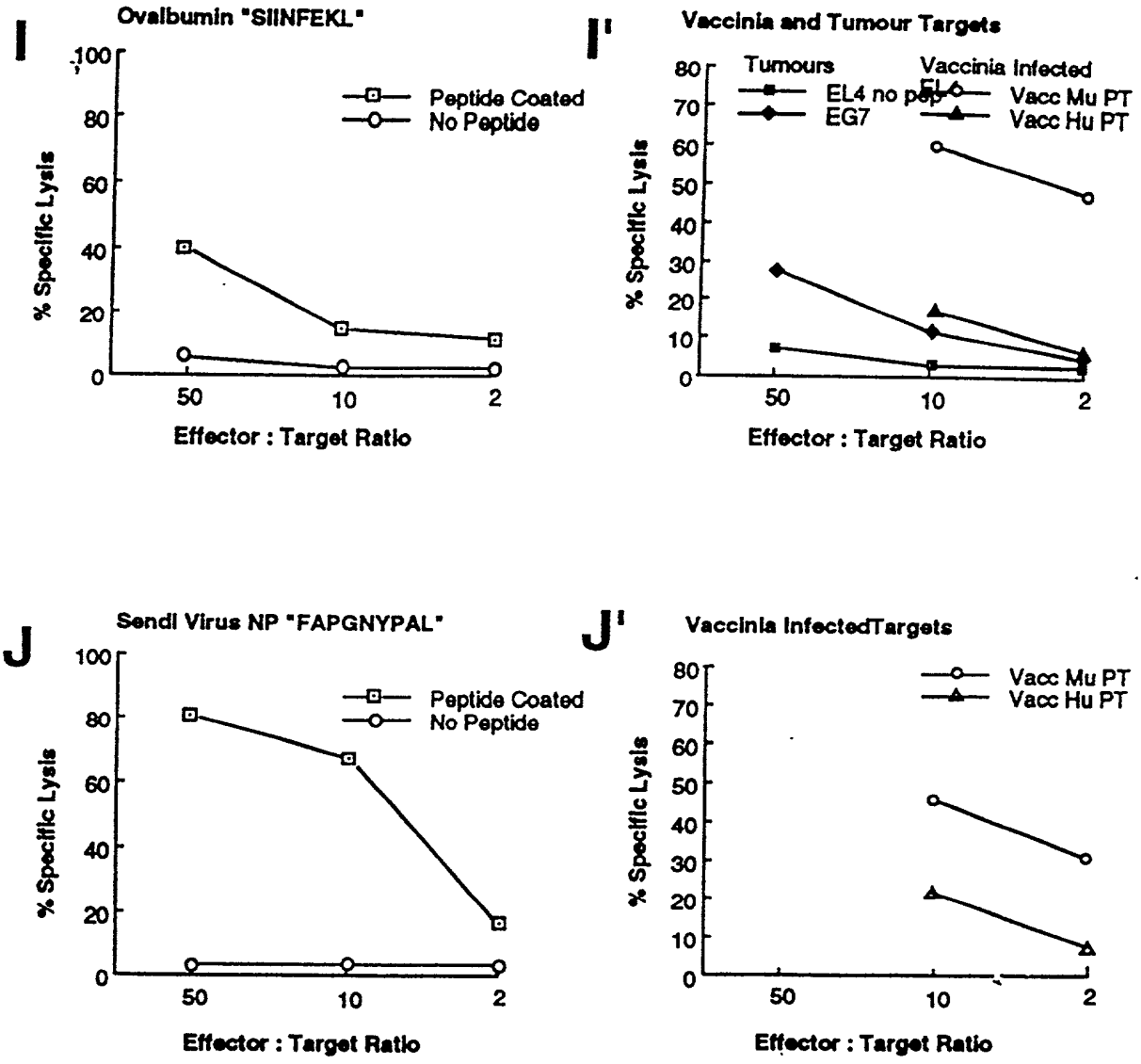

I
Ovalbumin "SIINFEKL"
% Specific Lysis
Peptide Coated
No Peptide
Effector : Target Ratio
I'
Vaccinia and Tumour Targets
Tumours
EL4 no pep
EG7
Vaccinia Infected
EL
Vacc Mu PT
Vacc Hu PT
% Specific Lysis
Effector : Target Ratio
J
Sendi Virus NP "FAPGNYPAL"
% Specific Lysis
Peptide Coated
No Peptide
Effector : Target Ratio
J'
Vaccinia InfectedTargets
Vacc Mu PT
Vacc Hu PT
% Specific Lysis
Effector : Target Ratio

FIGURE 9

Attorney Docket No:

Declaration and Power of Attorney
For Patent Application
(US National Stage of
International Application)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I verily believe I am the original, first and sole inventor (if only one name is listed below) or a joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought, on the invention entitled: **Polyepitope Vaccines** which is described and claimed in international application No. PCT/AU95/00461 filed 27 July 1995 and was amended on which I have reviewed and for which I solicit a patent;

I hereby state that I have reviewed and understand the consents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

I hereby claim foreign priority benefits under Tile 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

PRIOR FOREIGN APPLICATION(S)			**Priority Claimed**	
PM 7079	Australia	27 July 1994	☒	☐
PN 1009	Australia	8 February 1995	☒	☐
			☐	☐
[Number]	**[Country]**	**[Day/Month/Year Filed]**	**Yes**	**No**

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and. insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112. I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

[Application Serial no]	**[Filing Date]**	**[Status: patented, pending, abandoned]**

I or we hereby appoint the following attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and request that all correspondence about the application be addressed to

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardize the validity of the application or any patent issuing thereon.

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